

Δ Np63 α functions as both a positive and a negative transcriptional regulator and blocks *in vitro* differentiation of murine keratinocytes

Kathryn E King¹, Roshini M Ponnampersuma¹, Toshiharu Yamashita², Takashi Tokino², Lela A Lee³, Marian F Young⁴ and Wendy C Weinberg^{*,1}

¹Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20892, USA; ²Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan; ³University of Colorado School of Medicine and Denver Health Medical Center, Denver, CO 80262, USA; ⁴National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD 20892, USA

Δ Np63 is overexpressed in squamous carcinomas where it is associated with proliferation and is believed to enhance cell growth by blocking p53-mediated transactivation. In normal epithelium, Δ Np63 α protein expression is abundant in basal cells and decreases with differentiation. To explore the biological consequences of Δ Np63 α overexpression in relation to squamous carcinogenesis, we evaluated its effect on normal squamous differentiation and p53 transactivation function in keratinocytes. Forced overexpression of Δ Np63 α in primary murine keratinocytes *in vitro* inhibits morphological differentiation induced by elevated extracellular [Ca²⁺], abrogates Ca²⁺-induced growth arrest, and blocks expression of maturation-specific proteins keratin 10 and filaggrin. This suggests that Δ Np63 overexpression in squamous carcinomas may serve to maintain the basal cell phenotype and promote cell survival. Δ Np63 α blocks transactivation of p53 responsive reporter constructs mediated by endogenous or exogenous p53 at 17 h postinfection, as expected. However, at 41 h, when p53-mediated transactivation is diminished, Δ Np63 α enhances transactivation of these reporter constructs by 2.2–12-fold over control. Maximal Δ Np63 α -induced transactivation requires intact p53 responsive elements, but is independent of cellular p53 status. This positive transcriptional function of Δ Np63 α appears to be cell-type specific, as it is not observed in primary dermal fibroblasts or Saos-2 cells. These findings support Δ Np63 α as a master regulator of keratinocyte differentiation, and suggest a novel function of this protein in the maintenance of epithelial homeostasis.

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Introduction

The p53 homologues, p63 and p73, were identified on the basis of their structural similarity to the p53 tumor suppressor protein (Kaghad *et al.*, 1997; Osada *et al.*, 1998; Senoo *et al.*, 1998; Trink *et al.*, 1998; Yang *et al.*, 1998, 2002; Lee *et al.*, 1999; Bamberger and Schmale, 2001; Irwin and Kaelin, 2001; Moll *et al.*, 2001). Both p63 and p73 are expressed as multiple isoforms arising from alternative promoter usage and c-terminal alternative splicing (Osada *et al.*, 1998; Senoo *et al.*, 1998; Yang *et al.*, 1998; Yang and McKeon, 2000; Bamberger and Schmale, 2001; Moll *et al.*, 2001; Yang *et al.*, 2002). TA isotypes contain a transactivation domain homologous to that of p53 and can mimic p53 transactivation function, whereas Δ N isotypes lack this domain and block reporter activity mediated by p53 and TA-p63 (Yang *et al.*, 1998).

The p63 gene is critical for the development of stratified squamous epithelium, and Δ Np63 α is the predominant isotype within this tissue (Mills *et al.*, 1999; Parsa *et al.*, 1999; Yang *et al.*, 1999). Recent studies of squamous cell cancers demonstrate amplification of the p63 gene locus and overexpression of p63, particularly Δ Np63, at the protein and mRNA levels (Crook *et al.*, 2000; Hibi *et al.*, 2000; Yamaguchi *et al.*, 2000; Glickman *et al.*, 2001; Senoo *et al.*, 2001; Hu *et al.*, 2002; Pelosi *et al.*, 2002). This overexpression is primarily associated with the proliferative compartment (Parsa *et al.*, 1999; Pelosi *et al.*, 2002; Reis-Filho *et al.*, 2002), but its relevance remains unclear. Targeted overexpression of Δ Np63 α blocks UVB-induced apoptosis of transgenic mouse epidermal keratinocytes (Liefer *et al.*, 2000), a p53-dependent process (Ziegler *et al.*, 1994), and this and other studies suggest that overexpression of Δ Np63 α could contribute to aberrant growth regulation associated with neoplastic development or progression in epithelial tissues by blocking p53 function (Crook *et al.*, 2000; Liefer *et al.*, 2000). In murine keratinocytes, loss of p53 promotes the progression of experimentally induced papillomas to undifferentiated squamous cell carcinomas (Kemp *et al.*, 1993; Weinberg *et al.*, 1994).

Many studies aimed at defining the functions of specific p63 isotypes have been performed in

*Correspondence: WC Weinberg, Center for Biologics Evaluation and Research, FDA, 29 Lincoln Drive, HFM-564, Bldg 29B, Room 3NN04, Bethesda, MD 20892-4555, USA; E-mail: weinberg@cber.fda.gov

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nonepithelial assay systems, yet endogenous Δ Np63 expression is restricted primarily to epithelial tissues (Osada *et al.*, 1998; Yang *et al.*, 1998; Bamberger and Schmale, 2001; Dellavalle *et al.*, 2001; Di Como *et al.*, 2002; Serber *et al.*, 2002). Therefore, we utilized primary murine epidermal keratinocytes as a model of squamous epithelium to challenge the effect of Δ Np63 α overexpression on growth regulation, differentiation potential, and p53 function in relation to squamous carcinogenesis. In this study, we demonstrate that overexpression of Δ Np63 α dramatically blocks morphological differentiation and the corresponding differentiation-specific protein expression induced by elevated $[Ca^{2+}]$ *in vitro*, while at the same time abrogating differentiation-associated growth arrest. In addition to its ability to block transactivation by both exogenous and endogenous p53, we show that Δ Np63 α can activate transcription from p53 responsive reporter constructs in keratinocytes. This transactivation is cell-type specific, time- and consensus sequence-dependent and independent of cellular p53 status. These results demonstrate that Δ Np63 α can function as a master regulator of the keratinocyte differentiation program, and suggest a novel mechanism for Δ Np63 α action.

Results

In normal epithelium, endogenous Δ Np63 α is highly expressed in the proliferating compartment (Yang *et al.*, 1998; Parsa *et al.*, 1999). *In vitro*, growth arrest and induction of keratinocyte differentiation can be triggered by elevating extracellular $[Ca^{2+}]$ to >0.10 mM (Yuspa *et al.*, 1989). At 24 h following the introduction of the calcium trigger, we observed a decline in endogenous Δ Np63 α protein expression (Figure 1a), consistent with reported observations *in vivo* (Yang *et al.*, 1998; Parsa *et al.*, 1999). To model the Δ Np63 overexpression observed in human squamous cell carcinomas, we used adenoviral transduction to introduce Δ Np63 α (Ad- Δ Np63 α), or β -galactosidase (Ad- β -gal) as a control, into cultured murine keratinocytes. Effects on morphology, cell cycle regulation and biochemical responses were evaluated under conditions that normally maintain keratinocyte proliferation or induce differentiation. As shown in Figure 1b, overexpression of Ad- Δ Np63 α inhibits the onset of differentiation-associated squamous morphology that is observed in Ad- β -gal control keratinocytes following exposure to 0.12 mM Ca^{2+} . Δ Np63 α overexpression also abrogates the growth arrest normally associated with differentiation, as determined by FACS analysis of BrdU pulsed keratinocytes. As shown in Figure 2a, the S phase population of control keratinocytes expressing Ad- β -gal decreased on average 43% following 24 h of exposure to 0.12 mM Ca^{2+} , whereas no parallel reduction in the S phase population was observed in keratinocytes overexpressing Ad- Δ Np63 α under identical conditions. These changes in the calcium response of keratinocytes overexpressing Ad- Δ Np63 α , as assessed

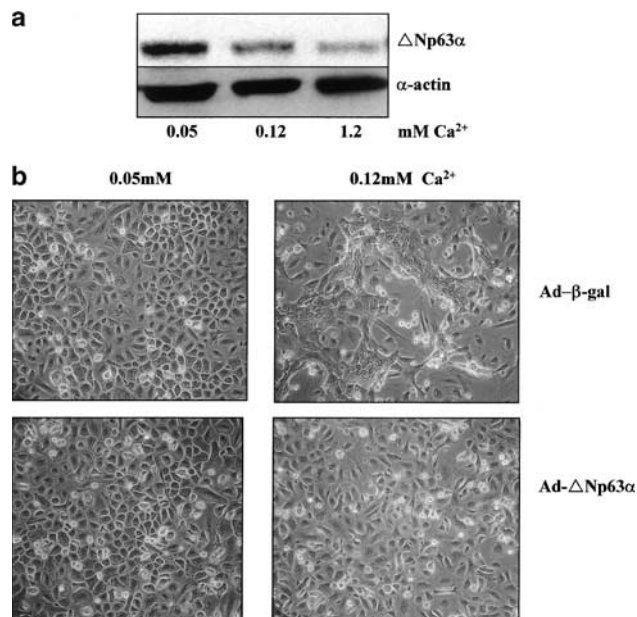


Figure 1 Δ Np63 α overexpression inhibits the onset of squamous morphology in keratinocytes. (a) At 24 h following the induction of differentiation via increasing extracellular $[Ca^{2+}]$, endogenous levels of Δ Np63 α were evaluated by Western blotting of soluble lysates from proliferating or differentiating keratinocytes. Δ Np63 α protein levels decrease with differentiation. (b) Keratinocytes were infected with adenovirus encoding Δ Np63 α or β -galactosidase. At 17 h postinfection, cultures were either maintained in medium containing 0.05 mM Ca^{2+} or induced to differentiate by increasing extracellular $[Ca^{2+}]$ to 0.12 mM. Phase morphology of keratinocytes 26 h following induction of differentiation. Keratinocytes overexpressing Δ Np63 α retain the phenotype of proliferating cells under differentiating culture conditions

by morphology and cell cycle analysis, are associated with altered expression profiles of differentiation-specific proteins. Western analysis demonstrated that overexpression of Ad- Δ Np63 α blocks Ca^{2+} -induced expression of keratin 10, a spinous layer protein, as well as the granular layer protein filaggrin (Figure 2b). No effect was observed on the induction of keratin 1, which is expressed prior to keratin 10. Expression of filaggrin remained blocked throughout the time course evaluated, whereas levels of keratin 10 began to increase at later timepoints, corresponding with reduced levels of adenovirally driven Δ Np63 α expression (Figure 2b). This suggests that the decrease in Ad- Δ Np63 α levels is permissive for keratin 10 expression. Taken together, these data suggest a role for overexpressed Δ Np63 α in squamous carcinogenesis in maintaining a basal cell phenotype and promoting cell survival.

Previous studies have suggested that Δ Np63 overexpression may be oncogenic because of its ability to block activity of the tumor suppressor p53 (Yang *et al.*, 1998; Crook *et al.*, 2000; Liefer *et al.*, 2000). Therefore, we examined the effect of Δ Np63 α overexpression on transactivation mediated by both endogenous and exogenous p53 in keratinocytes. Endogenous p53 activity has been shown to increase when keratinocytes are induced to differentiate by exposure to elevated

a

treatment	+ Ad- β -gal (control)			+ Ad- Δ Np63 α		
	expt. #	% S phase	s.d.	expt. #	% S phase	s.d.
0.05mM Ca ²⁺	1	14.97 (4)	1.08	1	12.32 (4)	0.90
	2	15.87 (6)	1.53	2	11.40 (6)	2.72
	3	18.97 (6)	1.42	3	13.36 (6)	1.29
	mean	16.81	2.19	mean	12.36	1.99
0.12mM Ca ²⁺	1	8.03 (3)	0.87	1	10.83 (4)	2.44
	2	11.20 (6)	1.40	2	18.42 (6)	2.30
	3	9.12 (6)	1.64	3	11.39 (6)	0.87
	mean	9.73	1.86	mean	13.89	4.00

b

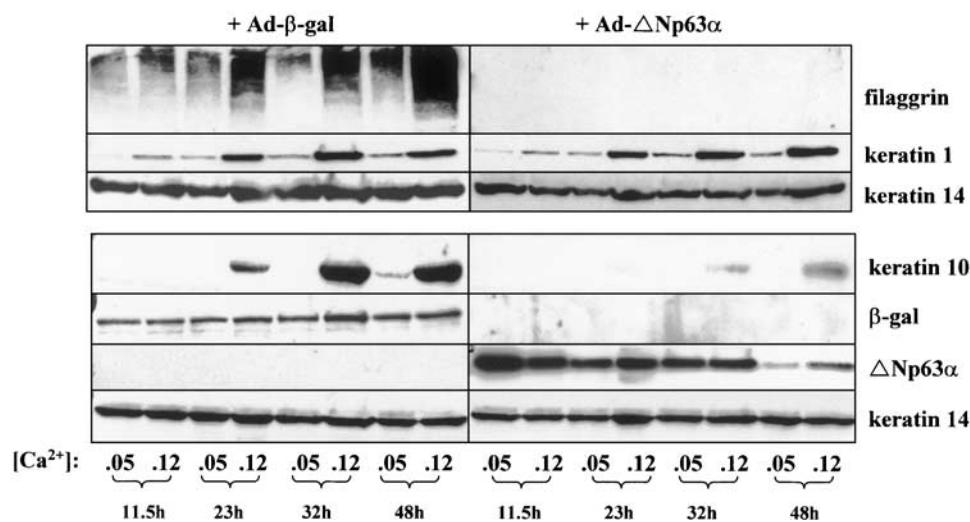


Figure 2 Δ Np63 α overexpression blocks differentiation-associated growth arrest and differentiation-specific protein expression. Keratinocytes were infected with adenovirus encoding Δ Np63 α or β -galactosidase. At 17 h postinfection, cultures were either maintained in medium containing 0.05 mM Ca²⁺ or induced to differentiate by increasing extracellular [Ca²⁺] to 0.12 mM for 24 h. **(a)** Cultures were pulsed with 10 μ M BrdU for the final 4 h prior to harvesting, and the S phase population of each sample was determined using FACS analysis. Values in parenthesis indicate number of replicates. Data are presented as mean \pm s.d. for each of three independent experiments. As shown in bold, the average S-phase population in β -gal control cultures decreased by 43% following exposure to 0.12 mM Ca²⁺. No reduction in the S phase population was observed in keratinocytes overexpressing Δ Np63 α . **(b)** Whole-cell lysates were harvested 11.5, 23, 32 or 48 h post-Ca²⁺ trigger. Top panel: membranes were probed sequentially with antibodies directed to filaggrin, keratin 1, and keratin 14. Bottom panel: replicate Western blots of lysates described above were probed sequentially with antibodies directed to keratin 10, β -galactosidase, Δ Np63 α , and keratin 14. For each antibody, both treatment groups were exposed to ECL reagent for the same length of time. Induction of differentiation-specific proteins filaggrin and keratin 10, but not keratin 1, is blocked by Δ Np63 α overexpression

[Ca²⁺], and maximal activity is achieved at extracellular Ca²⁺ levels of 1.2 mM (Weinberg *et al.*, 1995). Since increases in p53 activity are inversely correlated with Δ Np63 α levels (see Figure 1a), we speculated that endogenous p53 activity is modulated by Δ Np63 α . Therefore, we examined the effect of forced overexpression of Δ Np63 α on the transactivation of p53 responsive reporters. Consistent with previous observations (Weinberg *et al.*, 1995), low levels of endogenous p53 transcriptional activity are detectable in proliferating Ad- β -gal control cultures, as detected with the p53-responsive PG₁₃luc reporter construct, and these levels increase in the presence of 1.2 mM Ca²⁺ (Figure 3a). Similar results were obtained using the p53 responsive BDS-2 reporter construct (Figure 3b). Surprisingly, no inhibition of reporter gene activity was observed at the

same timepoint in cultures overexpressing Δ Np63 α under either Ca²⁺ concentration (Figure 3a, b). Under low Ca²⁺ conditions (0.05 mM), Δ Np63 α overexpression caused a 2.96-fold increase in PG₁₃luc and an 11.7-fold increase in BDS-2 reporter activity relative to Ad- β -gal controls. In 1.2 mM Ca²⁺, a 2.2-fold increase in PG₁₃luc and a 10.7-fold increase in BDS-2 reporter gene activity was seen in cultures expressing Ad- Δ Np63 α as compared to Ad- β -gal controls (Figure 3a, b). The greater activity levels observed in 1.2 mM compared to 0.05 mM Ca²⁺ can be explained by increases in the adenovirally driven Δ Np63 α protein expression (not shown). Transactivation with Δ Np63 α was observed regardless of whether the adenovirus was introduced prior to or after transfection with the reporter construct, and was also seen following cotransfection of a Δ Np63 α expression

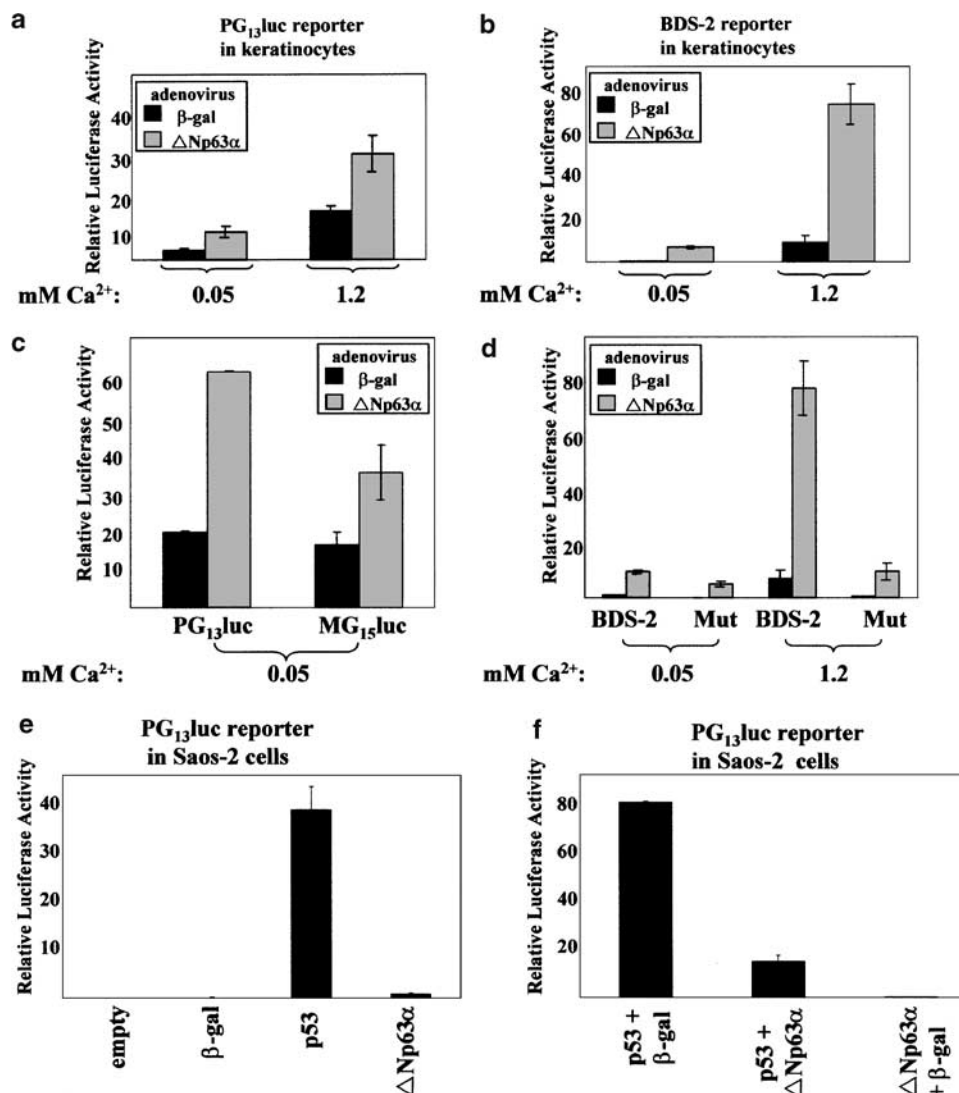


Figure 3 Δ Np63 α transactivates p53 responsive reporter constructs in keratinocytes. (a/b) Primary mouse keratinocytes in 0.05 mM Ca²⁺-containing medium transfected with PG₁₃luc or BDS-2 reporter constructs and infected with adenoviruses encoding β -gal or Δ Np63 α were subsequently maintained in standard culture medium containing 0.05 or 1.2 mM Ca²⁺. Keratinocytes were harvested for luciferase assays at 41 h following infection. All transactivation studies presented represent the mean of triplicate assays \pm s.d. and are representative of a minimum of three independent experiments. Overexpression of Δ Np63 α resulted in increased luciferase activity. (c/d) Primary mouse keratinocytes were transfected with reporter vectors containing intact (PG₁₃luc, BDS-2) or their respective mutated (MG₁₅, mut) p53-responsive sequences as noted, and infected with β -gal or Δ Np63 α adenoviruses. Cultures were maintained in standard culture medium containing either 0.05 or 1.2 mM Ca²⁺ and harvested 41 h postadenoviral infection. Results represent the mean of triplicate assays \pm s.d. and are representative of a minimum of two independent experiments. Intact p53-responsive sequences contribute to maximal luciferase activity. (e) Saos-2 cells transfected with PG₁₃luc and infected with control, β -gal, p53, and Δ Np63 α adenoviruses were harvested for luciferase activity at 41 h postinfection. Transactivation of reporter genes by Δ Np63 α is not apparent in this cell type. (f) Saos-2 cells were transfected with PG₁₃luc, then coinfecting with a 1 : 1 ratio of the following adenoviruses: p53/ β -gal, p53/ Δ Np63 α or Δ Np63 α / β -gal. Cells were harvested at 41 h postinfection. Δ Np63 α blocks p53-mediated transactivation

vector with PG₁₃luc (results not shown). To address whether the p53 consensus sequence is required for this activity, reporter constructs encoding mutations in the p53 responsive elements (Kern *et al.*, 1991; Hermeking *et al.*, 1997) were introduced into keratinocytes overexpressing Ad- Δ Np63 α or Ad- β -gal. These constructs showed diminished activity relative to constructs containing the wild-type consensus sequence (Figure 3c, d), thus an intact p53 consensus binding sequence participates in maximal Δ Np63 α -mediated transactivation activity.

As a result of the unexpected effect of Δ Np63 α on transactivation of reporter genes in keratinocytes, the experiment was repeated in Saos-2 cells as a negative control. Consistent with previously reported data (Yang *et al.*, 1998), Ad- Δ Np63 α did not activate expression from PG₁₃luc in this cell line, while transactivation was induced by Ad-p53 (Figure 3e). Coinfection of Saos-2 cells with adenoviruses encoding Δ Np63 α and p53 confirmed that Δ Np63 α blocks transactivation of PG₁₃luc by exogenous p53 in Saos-2 cells, as described previously (Figure 3f). The difference in the transactiva-

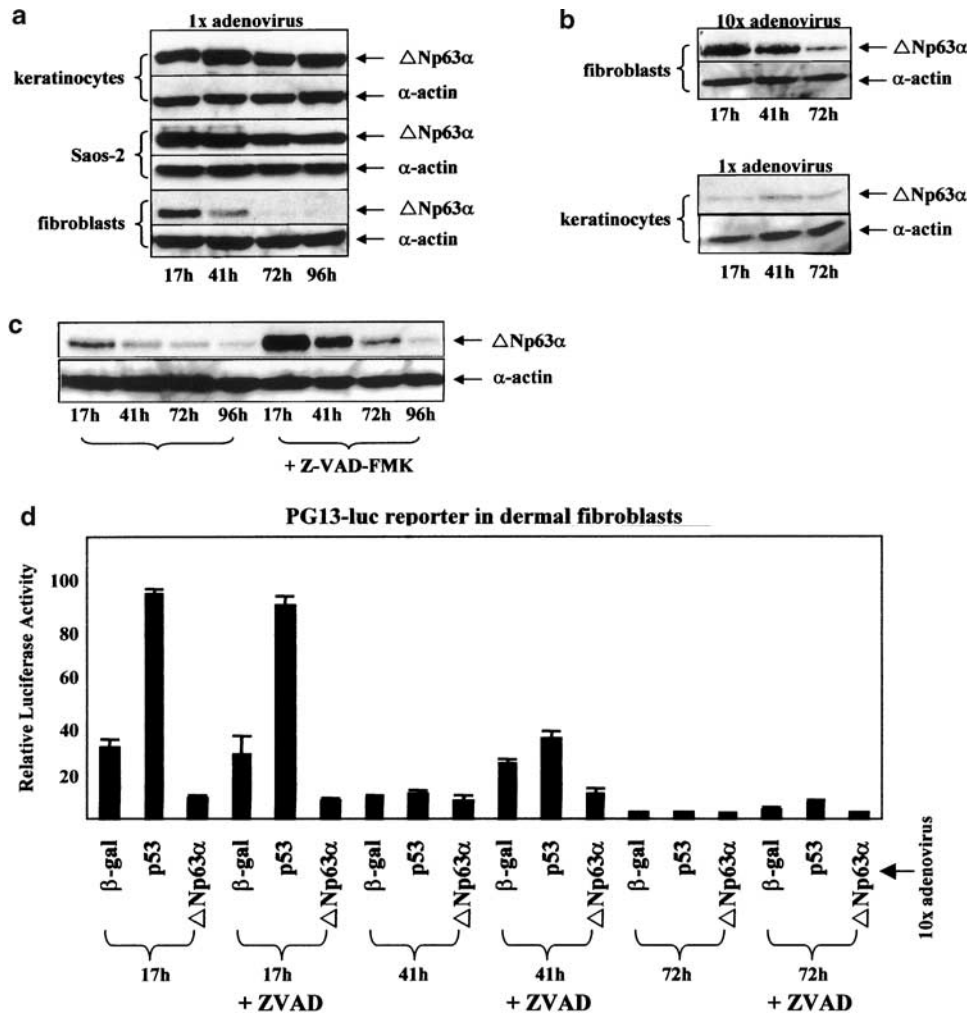


Figure 4 Transactivation by Δ Np63 α is cell-type specific. (a–c) Keratinocytes, Saos-2 cells, and dermal fibroblasts were infected with adenovirus encoding Δ Np63 α or β -gal and harvested at 17, 41, 72 and 96 h postinfection for Western blot analysis. Membranes were probed with antibodies to p63 (4A4) and α -actin. (a) All panels were exposed to ECL reagent for the same length of time. Adenoviral Δ Np63 α levels expression levels are similar in keratinocytes and Saos-2 cells, but are lower in fibroblasts. (b) Fibroblast cultures were infected with 10 \times higher adenoviral titer than keratinocytes, and panels were exposed for the same length of time. Increasing adenoviral titer 10-fold enhances Δ Np63 α protein expression in fibroblasts. (c) Fibroblasts were infected with Δ Np63 α adenovirus and incubated with and without Z-VAD-FMK (40 μ g/ml) up to timepoints indicated. Z-VAD-FMK stabilizes Δ Np63 α expression in fibroblasts. (d) Fibroblasts were transfected with PG13-luc and infected with adenoviruses encoding β -gal, p53, and Δ Np63 α . Cells were maintained in the presence or absence of Z-VAD-FMK (40 μ g/ml) and harvested 17, 41 and 72 h postinfection for luciferase assays. Even with enhanced expression levels, no reporter activity was seen with Δ Np63 α overexpression in fibroblasts

tion activity driven by Δ Np63 α between these cell types is not because of varying Ad- Δ Np63 α expression levels, as Saos-2 cells and keratinocytes express similar levels of Δ Np63 α following adenoviral transduction (Figure 4a).

To confirm the cell-type-specific nature of Ad- Δ Np63 α -mediated transactivation, we assayed primary fibroblasts derived from the same tissue as keratinocyte preparations, thus alleviating differences because of species specificity or acquired genetic changes reflecting the long-term culture or origin of Saos-2 cells. Infecting fibroblasts at the same adenoviral titers used for keratinocytes and Saos-2 cells resulted in relatively lower initial levels and less stable expression of Ad- Δ Np63 α (Figure 4a). Increasing the titer 10-fold boosted expression levels of Ad- Δ Np63 α in the fibroblasts above those observed in the other cell types (Figure 4b).

Δ Np63 α has been shown to be degraded by caspases (Ratovitski *et al.*, 2001). Therefore to further enhance Δ Np63 α protein expression fibroblasts were cultured in the presence of the caspase inhibitor Z-VAD-FMK, which extended detectable expression of Δ Np63 α through 72 h postinfection (Figure 4c). Regardless of Ad- Δ Np63 α expression levels achieved, transactivation of the PG13-luc reporter construct was never observed in fibroblasts at any timepoint evaluated (Figure 4d). Thus, the lack of transactivation in fibroblasts is not simply because of differing expression levels in this cell type, but more likely reflects cellular background and cell-type-specific functional differences in Δ Np63 α activity.

To determine whether Δ Np63 α is unique among the p63 isoforms in its differential activity across cell types, similar assays were performed with the TAp63 α and

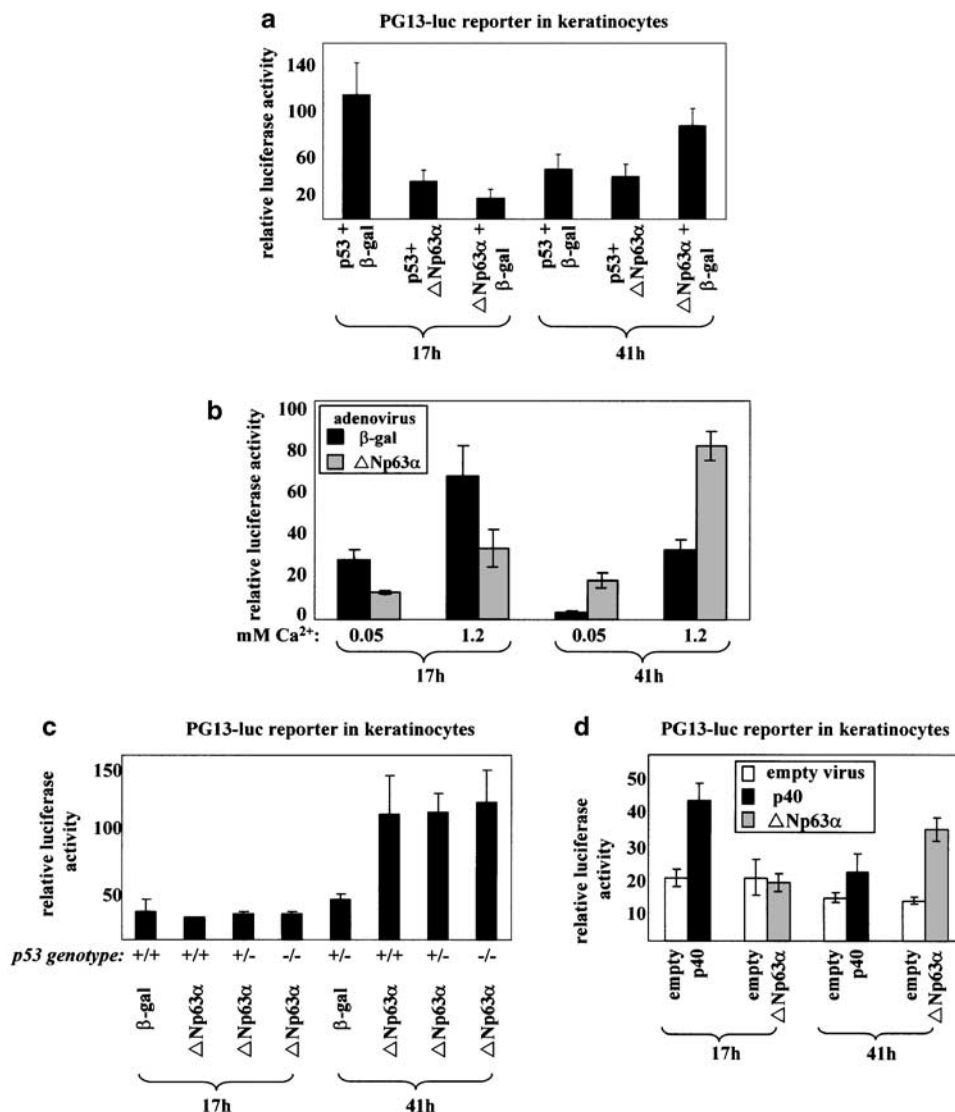


Figure 5 Δ Np63 α exhibits dual functions in keratinocytes. (a) Keratinocytes transfected with PG₁₃luc were infected with a 1 : 1 ratio of the following adenoviruses: p53/ β -gal, p53/ Δ Np63 α , or Δ Np63 α / β -gal. Cells were harvested at 17 and 41 h postinfection. Results shown are the mean of triplicate assays \pm s.d. from three experiments. Δ Np63 α transactivation is delayed relative to that of p53 and Δ Np63 α blocks p53 activity prior to its transactivation function. (b) Keratinocytes in 0.05 mM Ca²⁺-containing medium transfected with the PG₁₃luc reporter construct and infected with Δ Np63 α or β -gal adenoviruses were subsequently maintained in medium containing either 0.05 or 1.2 mM Ca²⁺ for 17 and 41 h, then harvested for luciferase assays. Results presented represent the mean of triplicate assays \pm s.d. and are representative of a minimum of three independent experiments. Overexpression of Δ Np63 α blocks endogenous p53-mediated transactivation at early timepoints and upregulates reporter activity at later timepoints. (c) Proliferating keratinocytes from p53^{+/+} and p53^{-/-} mice were assayed as in (b). Δ Np63 α transactivation is independent of cellular p53 status. (d) Transactivation assays were performed in keratinocytes as in (b), using control vector or adenoviruses encoding p40 or Δ Np63 α . The carboxyl terminus is not required for the transactivation observed with Δ Np63 α .

TAp63 γ isotypes. TAp63 α failed to drive PG₁₃luc reporter gene expression in Saos-2 cells, presumably as a result of autoinhibition arising from the α -terminus (Yang *et al.*, 1998, 2002; Serber *et al.*, 2002). Consistent with results in Saos-2 cells, TAp63 α did not transactivate the p53-responsive reporter PG₁₃luc in keratinocytes (results not shown). The p63 isotype TAp63 γ , which transactivates PG₁₃luc in Saos-2 cells, also induced transactivation in keratinocytes (results not shown).

As shown in Figure 3, Ad- Δ Np63 α exhibits strong transactivational activity in keratinocytes at 41 h post-

infection, but no blocking effect on endogenous p53 activity is apparent. To clarify whether the blocking effect of Ad- Δ Np63 α on endogenous p53 activity was masked by its strong transactivational activity, PG₁₃luc transfected keratinocytes were infected with adenovirus encoding p53 or Δ Np63 α together or in combination with Ad- β -gal control. These experiments revealed that Ad-p53 and Ad- Δ Np63 α exhibit distinct time courses of transactivational activity in this system, and explain why blocking of p53 by Δ Np63 α was not initially observed (Figure 5a). Transactivation of PG₁₃luc by Ad-p53 is evident as early as 17 h postinfection and declines by

41 h postinfection, while transactivation by Ad- Δ Np63 α is present at 41 h, but is not yet apparent at 17 h. Blocking of Ad-p53-mediated transactivation by Ad- Δ Np63 α can be discerned at 17 h, a time prior to the onset of Ad- Δ Np63 α -mediated transactivation (Figure 5a). When the initial assay to evaluate the effect of Ad- Δ Np63 α on endogenous p53 activity (Figure 3a, b) was repeated at 17 h, it became clear that Ad- Δ Np63 α also blocks transactivation induced by endogenous p53 at this earlier timepoint (Figure 5b). This dominant-negative activity subsides by 41 h postinfection, when transactivation of the PG₁₃luc reporter vector becomes apparent in the cells overexpressing Ad- Δ Np63 α (Figure 5b). Thus, p53 and Δ Np63 α exhibit differential time courses of transactivation activity and Δ Np63 α does block p53 transactivation function at early timepoints in this assay.

To further explore the relation between p53 and Δ Np63 α -induced transactivation in keratinocytes, assays were performed in keratinocytes of varying p53 gene dosages. No difference was seen in the timing or degree of transactivation in keratinocytes from p53^{+/+}, p53^{+/-} and p53^{-/-} mice following introduction of Ad- Δ Np63 α (Figure 5c). Thus, cellular p53 status neither interferes with nor contributes to transactivation modulated by Δ Np63 α in keratinocytes.

The α -carboxy terminus of TAp63 α has been implicated in the autoinhibition of transactivational activity mediated by TAp63 α (Serber *et al.*, 2002; Yang *et al.*, 2002). To determine whether this domain also influences transactivation mediated by Δ Np63 α , we introduced p40, a Δ Np63 isotype that is truncated immediately after the oligomerization domain (Trink *et al.*, 1998). In the absence of the α carboxyl terminus, Δ Np63 induces PG₁₃luc transactivation at 17 h post-infection (Figure 5d), potentially reflecting loss of a negative regulatory domain within the carboxyl terminus (Yang *et al.*, 1998; Serber *et al.*, 2002). Additionally, this demonstrates that the α -terminus is not required for Δ Np63-mediated transactivation in keratinocytes.

Discussion

Genetic deletion studies demonstrated that the p63 gene is critical for the development of squamous epithelium, but the relative contribution of the individual isotypes is still unclear (Mills *et al.*, 1999; Yang *et al.*, 1999). Δ Np63, the predominant isotype in proliferating epithelium, is overexpressed in squamous cell carcinomas (Crook *et al.*, 2000; Hibi *et al.*, 2000; Senoo *et al.*, 2001; Hu *et al.*, 2002). To determine the functional significance of this overexpression, we adenovirally introduced Δ Np63 α into keratinocytes of squamous epithelium and evaluated the capacity of these cells to respond to differentiation-inducing Ca²⁺ signals *in vitro*. Overexpression of Δ Np63 α dramatically inhibited the onset of squamous morphology and blocked Ca²⁺-induction of differentiation-specific proteins keratin 10 and filaggrin (Figures 1b, 2b). Additional evidence of a role

for Δ Np63 α in the maintenance of the proliferative cell population is derived from cell cycle analyses, in which Δ Np63 α overexpression in keratinocytes was shown to abrogate the Ca²⁺-induced reduction in the S phase population. The present findings suggest that a decrease in Δ Np63 α expression levels is necessary for normal differentiation to occur and that overexpression of Δ Np63 within tumors may maintain the proliferative state of neoplastic cells. This is consistent with previous studies of squamous cell carcinomas, in which overexpression of Δ Np63 is localized to the undifferentiated cells and is removed from areas of terminal differentiation (Parsa *et al.*, 1999; Reis-Filho *et al.*, 2002).

Both p73 and p53 null mice develop with no apparent epidermal abnormalities (Donehower *et al.*, 1992; Weinberg *et al.*, 1995; Yang *et al.*, 2000), suggesting that it may be the balance between the Δ N and TA isotypes of p63 that is critical for normal keratinocyte growth regulation. The blocking effect of Δ Np63 α on TAp63-mediated transcription may contribute to the aberrant growth arrest observed in these studies. For example, the CDK inhibitor p21^{WAF1} was initially identified as the product of a p53-responsive gene, however elevation of p21^{WAF1} levels in conjunction with differentiation-associated growth arrest is independent of p53 (Missero *et al.*, 1995; Weinberg *et al.*, 1995). We speculate that p21^{WAF1} is a target of transactivation by endogenous TAp63, and that this transactivation is blocked in the presence of elevated levels of Δ Np63 α .

The undeveloped epidermis and perinatal lethality of p63 null mice preclude the examination of keratinocyte growth regulation and differentiation in a cellular background devoid of p63, and the development of conditional transgenic models will be necessary to better understand the *in vivo* roles of each of the isotypes. The present studies offer important data on the biological effects of the Δ Np63 α isotype and the interplay between the family members in a relevant cellular context. These cell culture studies also help to clarify the importance of the sequential relation between Δ Np63 α expression levels and the differentiation trigger. In the experiment shown in Figure 2b, the Ca²⁺ trigger was introduced at a time when high levels of exogenous Δ Np63 α protein were already expressed. When the differentiation trigger was introduced concomitant with the cDNA for Δ Np63 α but prior to elevated protein levels, the morphological effect on differentiation was still apparent but less pronounced (results not shown). These findings suggest that induction of Δ Np63 α expression coincident with or subsequent to the earliest differentiation trigger may not be sufficient to block the consequential differentiation response, and may explain why transgenic mice with Δ Np63 α targeted to the epidermis with the loricrin promoter lack an apparent differentiation phenotype (Liefer *et al.*, 2000).

Beyond blocking keratinocyte differentiation and p53-mediated transactivation, we demonstrate that Δ Np63 α is capable of positively regulating gene transcription in epidermal keratinocytes, but not in

dermal fibroblasts or Saos-2 cells. Consistent with our findings, the selective transactivation of p53-responsive genes by tetracycline-regulated Δ Np63 α was recently noted in the epithelial H1299 carcinoma cell line (Dohn *et al.*, 2001). Our studies with primary fibroblasts derived from the same skin samples as the keratinocytes eliminate the possibility that the differences observed between cell types are because of species variations, acquired genetic alterations, or variable expression levels. The restricted pattern of p63 expression suggests that it provides a tissue-specific function relevant to the development and maintenance of the basal cell phenotype. The cell-type-specific transactivation by Δ Np63 α may participate in and reflect this role.

It has been proposed that the amino terminal 26 amino acids of Δ Np63 encode a novel transactivation domain (Dohn *et al.*, 2001; Duijf *et al.*, 2002), supporting a direct mechanism of gene regulation by Δ Np63. Consistent with this model, the core DNA binding domain of p63, which is shared across all p63 isoforms, is capable of binding multiple p53 responsive consensus sequences (Klein *et al.*, 2001), and we showed that mutations in the p53 consensus sequence diminish transactivation levels. However, the temporal delay in transactivation observed with Δ Np63 α in keratinocytes (Figure 5a) suggests that induction of additional cofactors not available in other cell types may be required. Whether such cofactors cooperate in transactivation by blocking negative regulators of gene transcription or by alleviating inhibition because of the α -carboxy terminus, as suggested by the time course of transactivation by p40 (Figure 5d), remains unclear. In this context, it is interesting to note that Δ Np63 has been shown to regulate positively transcription of VEGF as well as β -catenin-dependent transcription *via* interactions with additional proteins (Senoo *et al.*, 2002; Patturajan *et al.*, 2002).

The relative contributions of the positive and negative transcriptional regulatory functions of Δ Np63 α to keratinocyte growth regulation and differentiation remain to be elucidated. The activation by Δ Np63 α of the reporter constructs used in this study presumably reflects endogenous gene activity, and current efforts are focused on the identification of downstream targets of Δ Np63 α -mediated transactivation in this keratinocyte model. Microarray analyses have suggested several candidate genes, which will be the subject of future studies (KE King, M Gerdes and WC Weinberg, unpublished observations). Given the potential for interaction among p53 family members (Yang *et al.*, 1998; Davison *et al.*, 1999; Gaiddon *et al.*, 2001; Ratovitski *et al.*, 2001) and the ability of these family members to modulate transcription of multiple genes (Shimada *et al.*, 1999; De Laurenzi *et al.*, 2000; Dohn *et al.*, 2001; Moll *et al.*, 2001), changes in the relative ratios of p53 homologues could have strong implications for the expression patterns of target genes. The ultimate biological impact of Δ Np63 α overexpression is likely to reflect the specific cell populations targeted, the stage of differentiation of the expressing cell, as well as the balance of additional p53 family members.

Materials and methods

Cell culture

Primary keratinocytes and dermal fibroblasts were isolated from the skin of 1–2 day-old C57B1/6Ncr mice as described (Yuspa *et al.*, 1989; Weinberg *et al.*, 1994). Keratinocytes differing in p53 gene status were isolated from mice (+/+), (+/-), or (-/-) for a null mutation in the p53 gene on a mixed genetic background and genotyped as previously described (Weinberg *et al.*, 1994).

Freshly isolated keratinocytes were cultured in standard growth medium composed of minimal essential medium (SMEM) (Life Technologies, Gaithersburg, MD, USA), 8% chelexed fetal bovine serum (FBS) (Gemini BioProducts, Calabasas, CA, USA), 10 U/ml penicillin, and 10 μ g/ml streptomycin (Life Technologies), at a final concentration of 0.05 mM Ca^{2+} . Differentiation was induced by elevating extracellular $[\text{Ca}^{2+}]$ to >0.1 mM (Yuspa *et al.*, 1989). Fibroblasts were cultured in standard growth medium containing 1.2 mM Ca^{2+} . In some experiments, 40 μ g/ml of the caspase inhibitor Z-VAD-FMK was added to the medium (Enzyme Systems Products, Livermore, CA, USA). Saos-2 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM (Life Technologies, Gaithersburg, MD, USA) containing 10% newborn calf serum (BioWhittaker, Walkersville, MD, USA).

Adenoviruses

Adenovirus encoding Δ Np63 α was generated by ligating the complete coding region of a Δ Np63 α cDNA clone excised from pThioHis with *Xho*I into the *Sa*I site of the shuttle vector pcCMV-pLpA (Becker *et al.*, 1994; Lee *et al.*, 1999). cDNA orientation was verified via restriction analysis and appropriate constructs were cotransfected with pJM17 into HEK 293 cells by CaPO_4 precipitation for production of recombinant, replication-deficient adenovirus (Becker *et al.*, 1994). Transgene expression by plaque-purified viruses was confirmed via PCR using the following oligonucleotides: 5'-cgggtgggaggtc-tatataagcaga-3' and 5'-gggtgtcttcattcca-3', and positive viruses were propagated in 293 cells. Control adenoviruses were generated by cotransfection of pJM17 and the parent pcCMV-pLpA plasmid. Adenoviruses encoding p53 and β -galactosidase were constructed as described (Ishida *et al.*, 2000). Adenovirus encoding p40 and a matched control adenovirus (Hibi *et al.*, 2000) were the generous gifts of Dr D Sidransky.

Transient transfections and adenoviral infections

The p53-responsive luciferase reporter constructs PG₁₃luc and BDS-2, and the control constructs MG₁₅luc and BDS-2* that contain mutations in the p53 binding sequences (Kern *et al.*, 1991; Hermeking *et al.*, 1997), were generously provided by Dr B Vogelstein. For reporter gene assays, keratinocytes were plated at a density of $3\text{--}6 \times 10^6$ cells per 60 mm dish and cultured for 7 days prior to transfection. Fibroblasts and Saos-2 cells were plated 1 day prior to transfections at a density of 1×10^6 cells and 5.7×10^5 cells/60 mm dish, respectively. Transfections were performed with the Lipofectamine Reagent System (Life Technologies) according to the manufacturer's protocol but using 3 μ g of DNA and 4 μ l PLUS reagent per dish.

For adenoviral-mediated gene transduction, cells were incubated 60 min at 37°C in serum-free growth medium containing an equal MOI of the following adenoviral constructs: control vector, β -galactosidase, Δ Np63 α , p53 or p40. Following the incubation, the medium was aspirated and

replaced with standard growth medium. For transactivation assays, identical cultures were transfected with the reporter construct; immediately following transfection, cultures were infected with the adenovirus noted.

FACS analysis

Confluent cultures of primary murine keratinocytes were adenovirally infected with Ad- Δ Np63 α or Ad-lacZ at 1.5 days postplating. At 17 h postinfection, the medium was changed and cells were maintained for 24 h in 0.05 or 0.12 mM Ca^{2+} . Cultures were pulsed with 10 μM BrdU for the final 4 h. Cells were fixed in 70% ethanol for 24 h and incubated with an antibody to BrdU as per the manufacturer's protocol (Becton-Dickinson) followed by incubation with propidium iodide (5 $\mu\text{g}/\text{ml}$). The cell cycle distribution was analysed using Cell Quest software on a Becton Dickinson FACSCalibur. A total of three to six replicates were analysed per condition. Results from each of three independent experiments are presented as the mean % S phase population \pm s.d.

Luciferase assays and Western blotting

For transactivation assays, samples were harvested in Reporter Lysis Buffer (Promega Corp., Madison, WI, USA) at 17, 41, or 72 h postadenoviral infection. Protein concentrations were determined using the Protein Assay System (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were analysed using the Luciferase Assay System (Promega Corp., Madison, WI, USA), and luciferase activity was detected using a ML 3000 Microtitre plate luminometer (Dynatech Laboratories, Chantilly, VA, USA) or MicroLumat Plus Microplate Luminometer LB96 V (EG&G Berthold GmbH and Co., Bad Wilbad, Germany). Results shown are from representative

experiments and are presented as the mean of relative luciferase units from triplicate samples \pm s.d.

For Western analysis, soluble protein lysates and total cell lysates were harvested as described (Weinberg *et al.*, 1995; Veri *et al.*, 2001). Optitrans nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) were probed with the following antibodies: murine monoclonal anti-p63 (4A4, Santa Cruz Biotechnology, Santa Cruz, CA, USA); murine monoclonal anti- α -actin (Sigma Immuno Chemicals, St Louis, MO, USA); rabbit anti-keratins 1, 10, 14 and rabbit anti-filaggrin (BABC0, Richmond, CA, USA). Signal was detected using horseradish peroxidase linked anti-mouse or anti-rabbit Ig secondary antibody and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Abbreviations

β -gal, β -galactosidase; DMEM, Dulbecco's modified Eagle's medium; MOI, multiplicity of infection; Ad- Δ Np63 α , over-expression of Δ Np63 α mediated by adenovirus.

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